# THE RÔLE OF ANTIFERTILIZIN IN THE FERTILIZATION OF SEA-URCHIN EGGS

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## Introduction

In recent years several investigators (Frank, 1939; Tyler, 1939*a*, 1940; Southwick, 1939; Hartmann, Schartau and Wallenfels, 1940) have obtained from sperm of sea urchins and of mollusks, a substance that reacts with the fertilizin obtained from eggs, and which is therefore termed antifertilizin. The reaction is manifested by the following effects:—(1) When it is added to a solution of fertilizin, the sperm-agglutinating property is proportionately destroyed; (2) under appropriate conditions it forms a precipitate with fertilizin; (3) it agglutinates eggs of the same or closely related species; (4) it produces a precipitation membrane on the surface of the egg jelly. These four effects are evidently due to the same substance which is obtained as a sea-water extract of moderately heated or of frozen and thawed sperm.

Several other effects of sperm extracts have been described. In the keyhole limpet and in the abalone the extracts contain a lytic agent (Tyler, 1939*a*) which has the property of dissolving the membrane normally present on the unfertilized eggs of these species and which is much more heat-labile than the antifertilizin. The evidence does not as yet enable a decision to be made as to whether it is a distinct substance or a complex that is only active in combination with antifertilizin or a higher "polymer" of antifertilizin. A somewhat similar lytic action of macerated sperm on the egg membrane was reported in amphibia (Hibbard, 1928; Wintrebert, 1933) and of a sperm extract on the cell mass and membrane surrounding the egg of the rabbit (Yamane, 1935).

An agent that inhibited the activity of the spermatozoa was obtained by Southwick (1939) in the supernatant from centrifuging concentrated but not dilute sperm suspensions of the sea urchin. Identity of this agent with antifertilizin has not been established nor has the possibility been excluded that the effect is due to some simple agent such as increased acidity, $CO_2$, etc. A similar activity-inhibiting action has been

reported in sea urchins by Hartmann, Schartau and Wallenfels (1940) for a methyl alcohol extract of sperm that does not contain antifertilizin (the agglutinin-neutralizing agent). They also find that the extract neutralizes the stimulating effect of egg water on sperm activity and the similar action of echinochrome which they had earlier reported to be the sperm-activating agent in egg water. Since their findings with echinochrome have not been duplicated in other species (Tyler, 1939*b*; Cornman, 1941), and since they have not as yet disposed of the possibility suggested by Cornman that rise in pH might be responsible for their results, it would be desirable to have further evidence before the effect of their methanol sperm extract may be accepted without reserve.

Another effect reported by Hartmann, Schartau and Wallenfels (1940) in the sea urchin is the dissolving of the jelly coat of the egg by the action of sperm extract. They find in *Arbacia pustulosa* that addition of concentrated sperm extract or of live sperm causes the disappearance of the egg jelly and we have been able to confirm this in *Strongylocentrotus purpuratus*. But, according to our observations, this disappearance does not appear to be due to solution of the jelly. When sperm extract is added to a suspension of eggs there is formed on the surface of the jelly a precipitation membrane which, in concentrated extract, gradually increases in thickness and contracts until it reaches the surface of the egg. This precipitation membrane is evidently formed by interaction of the antifertilizin in the sperm extract with the jelly. The disappearance of the latter in concentrated extracts is most simply attributable to its incorporation in the precipitation membrane and to the considerably smaller volume it occupies in precipitated rather than in gel form. As the precipitation membrane contracts the egg may, particularly when disturbed, break out of it. When undisturbed it may contract to the surface of the egg from which it is then not readily distinguishable. The disappearance of the jelly under the influence of concentrated suspensions of live sperm is likewise attributable to combination with the antifertilizin on the sperm. There does not, then, appear to be, as yet, any necessity for the assumption of a jelly-dissolving agent in the sperm extract.

In the present work the term antifertilizin is applied to that substance derived from sperm that produces the effects listed in the first paragraph. A similar antifertilizin has been obtained from eggs (Tyler, 1940), but it will not enter into the present account. The principal question at issue here is whether or not the antifertilizin of sperm is concerned in the fertilization reaction. Several facts strongly favor the presumption that it is intimately involved. In the first place it is tissue

specific, being obtainable from no other tissues (Frank, 1939). It is, however, not very highly species-specific, since cross-reactions are obtained between species that do not cross-fertilize (Hartmann, et al., 1940). This would mean that it is not primarily responsible for the species-specificity of fertilization, but this does not exclude the possibility that it is an integral part of the fertilization process. Another fact favoring its involvement is that it is evidently present on the surface of the spermatozoön. Since, in solution, it reacts with fertilizin, it most likely is the substance on the spermatozoön that reacts in the agglutination of the sperm and therefore must form at least a part of the surface. Furthermore, fertilizin has been shown (Tyler, 1941) to serve as an aid to fertilization and may possibly be an essential agent in the process. Antifertilizin, since it reacts with it, would then be expected to have a complementary rôle.

For a direct test of the significance of antifertilizin, it would be desirable to remove it completely or partially from the sperm by some non-injurious method and to examine the fertilizing capacity of the treated sperm. We have been able, in the experiments reported here, to remove antifertilizin partially without appreciable damage to the sperm. This, as the results show, causes a considerable impairment in the fertilizing capacity of the sperm.

## Materials and Methods

Two species of sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus anamesus,* were employed in these experiments. Sperm and egg suspensions were prepared by removing the gonads to sea water and straining the shed sex cells through bolting cloth of appropriate mesh. The concentration of the sperm suspension was usually determined from the increase in volume after removal of the remains of the testes and is expressed as the percentage content of "dry" sperm.

The antifertilizin concentrations in the extracts were determined roughly by the intensity of the egg agglutination reaction and more accurately by the amount required to neutralize one unit (as defined by Tyler and Fox, 1940) of fertilizin (sperm agglutinin). In all the tests the pH of the solutions was checked and adjusted where necessary by means of the glass electrode.

The respiratory rate of the sperm was employed as an index of the extent of damage produced by the various treatments. The measurements were made in the Barcroft-Warburg apparatus with the cylindrical type of vessel previously described (Tyler and Humason, 1937). To

avoid possible effects of $CO_2$ and variation in pH, glycylglycine (Tyler and Horowitz, 1937) was used as a buffer in carbonate-free sea water.

### Removal of Antifertilizin

We found that antifertilizin could be removed from the sperm by slight acidification of the suspension and also by mild warming. The antifertilizin is obtained in the supernatant after centrifugation of an acidified sperm suspension but not in that of the control. When highly concentrated control sperm suspensions are centrifuged, particularly after aging, some antifertilizin may be obtained in the supernatant, as Southwick (1939) reported. This may mean that antifertilizin normally goes slowly into solution or that centrifugation of the concentrated suspensions involves some damage and consequent liberation of antifertilizin.

Antifertilizin is obtained from sperm suspensions acidified to pH 6 or lower. The more acid suspensions yield the more concentrated solutions. One experiment with *Strongylocentrotus* sperm may be cited. Samples of a 10 per cent suspension were acidified to pH 6, 5.6, 5.1, 4.5 and 3.5. After one hour the suspensions were brought back to the control pH (7.9) and centrifuged. The control supernatant was clear while those from the acidified suspensions were increasingly opalescent. Tested on eggs the control showed no reaction while the supernatants from the acidified samples gave precipitation membranes and agglutination which increased with increase in the degree of acidity to which the samples had been exposed. Tests of their ability to neutralize fertilizin gave the following approximate titres for the antifertilizin concentration in the supernatants of the acidified samples: ¼, ½, 1, 4 and 32 units respectively. The spermatozoa were all immotile in the sample that had been exposed to pH 3.5 and partly so in the pH 4.5 sample, while those exposed to the higher pH's showed considerable activity.

These results restricted then the investigation of the treatment required for the impairment of fertilizing capacity to the range between pH 5 and pH 6. A number of tests were run at various pH's within this range and with various times of exposure. All of these showed a considerable reduction in the fertilizing capacity of the treated sperm. Similar results were obtained by heating the sperm at 30° to 33° C. for 5 to 10 minutes. The data need not be presented here since only that part which was obtained along with the respiration measurements is of particular significance. In practically all of these tests the treated sperm were found to be quite active, although in general not as active as the controls. However, differences in activity of spermatozoa are hard to

estimate by direct observation. A more objective and quantitative method consists in measurement of the respiratory rate.

## Fertilizing Capacity and Respiratory Rate of Antifertilizin-Poor Sperm

Determinations were made, therefore, of the rate of oxygen uptake of the treated and control sperm along with tests of their respective fertilizing capacities. The results of five experiments are presented in Table I. Heat treatment was employed in one of these and acidification in the rest. The measurements were made in duplicate in each experiment, and both treated and control sperm were samples of the same original suspension. The control oxygen consumption values vary rather considerably in the different experiments. This variation is probably due to a number of factors such as error in initial determination of sperm concentration, variation in original condition of sperm, in its aging, etc. For the present purposes, however, this variation is of no particular significance, since comparison of treated and control sperm is made in each experiment. The duplicate runs in each experiment are in close agreement, which is to be expected since sperm suspensions can be quite accurately sampled and since the spermatozoa respire at a sufficiently high rate to make the instrumental errors relatively small.

In none of the experiments listed in Table I was the respiration of the treated sperm equal to that of the control. The highest values were 80 per cent of the control in experiments 1 and 5 and the lowest value was 25 per cent of the control in experiment 4. The treatment is, then, not entirely non-injurious to the sperm. However, a considerably greater impairment of fertilizing power results from the treatment. The fertilizing capacity of the treated sperm is listed in the last column of the table in terms of the amount required to give the same percentage fertilization, between 1 and 99 per cent, as is given by one part of the control sperm. These values are obtained from the results of inseminating samples of the same batch of eggs with serial dilutions of the control and treated sperm taken from the manometer vessels. The two figures for each experiment cover the range of variation. Thus, in the first experiment, the amount of treated sperm required to give the same percentage fertilization as the control is four to eight times the amount of the control sperm. For comparison, the next to the last column of the table gives the calculated amount of treated sperm that would have the same respiratory rate as one part of control sperm. This value is, in each experiment, considerably less than the value for the amount of sperm having a fertilizing capacity equal to one part of control sperm.

TABLE I

*Respiratory rate and fertilizing capacity of sea-urchin sperm after antifertilizin-liberating treatments.*

| Experiment | Treatment | $O_2$ consumption ($mm.^3$/hr./cc. of 1 per cent sperm) | | Amount of treated sperm equivalent in resp. rate to 1 part of control sperm | Amount of treated sperm equivalent in fertilizing capacity to 1 part of control sperm |
|---|---|---|---|---|---|
| | | Treated sperm | Control sperm | | |
| 1. *Strongylocentrotus* | 1½ hrs., at pH 5.3 | 14.3, 13.2 | 16.5, 17.9 | 1.25 parts | 4 to 8 parts |
| 2. " | 1 hr. at pH 5.1 | 2.46, 2.58 | 9.17, 9.47 | 3.7 parts | 64 to 128 parts |
| 3. " | 1 hr. at pH 5.5 | 8.12, 7.36 | 11.51, 10.72 | 1.44 parts | 16 to 32 parts |
| 4. " | 10 min. at 32° C. | 3.10, 3.35 | 13.19, 13.24 | 4.0 parts | >128 parts |
| 5. *Lytechinus* | 1 hr. at pH 5.4 | 0.54, 0.58 | 0.74, 0.67 | 1.26 parts | 16 to 32 parts |

In other words, there is, as a result of the treatment in each case, a very much greater reduction in the fertilizing power than in the respiratory rate.

It is evident, too, from the data that a considerable impairment of fertilizing power would be obtained following a treatment that resulted in no reduction in respiratory rate. That we have not, as yet, succeeded in finding the proper treatment which would give that result is not surprising in view of the variability of the sperm in the different experiments and the fact that the difference is rather small between treatments giving no effect and those giving a definite reduction in fertilizing capacity. The present results, however, suffice to show that an impairment of fertilizing power can be obtained that is disproportionately great when compared with the respiration of the sperm. This impairment cannot, then, be accounted for by a decrease in activity of the spermatozoa. It might possibly be interpreted in a rather complicated manner by the supposition that a corresponding fraction of the sperm are rendered non-respiring and non-fertilizing while the remainder have an increased respiratory rate. This would mean that the effect on the individual spermatozoa would be all or none and that mild treatment would have a stimulating effect on the respiration of the suspension. There is no evidence for this. The most reasonable interpretation is that the impairment of fertilizing capacity is correlated with the loss of antifertilizin which was shown to result from the treatment.

In the experiments described here antifertilizin is present in solution in the treated sperm suspension. To determine whether its presence might affect the results, antifertilizin was added to untreated sperm in the same or slightly greater amounts. This was found to have no effect on the fertilizing capacity of the sperm. On the other hand, when concentrated antifertilizin solutions are employed an inhibition of fertilization can be obtained, as Frank (1939) and Hartmann, Schartau and Wallenfels (1940) have shown. This inhibition occurs more readily when the eggs are first treated and is evidently due to the presence of the precipitation membrane that forms on the surface of the jelly. When this membrane is incomplete or torn the egg can be fertilized, as was previously reported in the case of treatment with the antifertilizin obtained from eggs (Tyler, 1940).

### Antigenicity of Antifertilizin and Action of Antisera

In order to obtain further information on the location of antifertilizin and on its rôle in fertilization, attempts were made to produce antibodies to it. Preliminary immunization experiments showed that high

titer agglutinins could be obtained in rabbits by the injection of whole sperm of the sea-urchin. Immunization with antifertilizin solutions likewise was found to induce the formation of specific agglutinins for the whole sperm as well as precipitins for the antigen in solution.

Antisera were produced against *Strongylocentrotus* and *Lytechinus* antifertilizin. The procedure and results in one experiment with *Lytechinus* follow. A solution of antifertilizin was prepared by extraction of a 25 per cent sperm suspension at pH 4.5 for two hours. The content of organic solid was determined on a sample that had been dialyzed against distilled water and was found to be between 15 and 20 mg. per cent. The rabbit was given seven intravenous injections totaling 23 cc. within a period of two weeks and was bled two weeks after the last injection. The antiserum showed by the ring test a precipitin titer of 8. Tested on a one per sperm suspension it showed an agglutinin titer of 512.

The production of agglutinins by injection of antifertilizin means not only that the substance is antigenic but is probably a surface antigen of the sperm. An examination of the agglutinates shows that the spermatozoa are stuck by their tails as well as by their heads. The antifertilizin, therefore, does not appear to be restricted to a particular location on the surface of the spermatozoön. It should also be noted here that extraction at pH 4.5 removes only a small part of the antifertilizin from the sperm since subsequent freezing and thawing or brief heating of the residue yields at least ten times the amount obtained in the acid extract. Also the acid-treated sperm are still agglutinable by antisera and by egg water.

The antigenicity of antifertilizin supports the view that it is a protein. Other evidence (to be presented in detail later) consists in its non-dialyzability, precipitation with $(NH_4)_2SO_4$, inactivation by heat and acidity, and the fact that it gives the common (xanthoproteic, Millon's and biuret) color tests.

The effect of the antiserum on fertilization was examined by insemination of eggs in its presence. Controls were run with normal rabbit serum. The sera were adjusted to sea-water salinity by the addition of an equal volume of concentrated (1.73 ×) sea water, and equal volumes of egg and sperm suspensions were added. In all cases where the sperm was diluted to the minimum for 100 per cent fertilization in the controls, no fertilization was obtained in the antiserum. With the dilutions of sperm employed, agglutination is greatly retarded and may even fail to occur in the antiserum. The spermatozoa have not then, to any great extent, been rendered inaccessible to the eggs by incorporation in

agglutinates. The inhibition of fertilization may therefore be considered to be due to the neutralization of antifertilizin on the sperm by its antibody in the antiserum.

## Discussion

The results presented here show that antifertilizin is involved in the fertilization process. In order to decide whether or not it has an indispensable rôle, one would like to have some more direct evidence such as the complete and reversible removal of antifertilizin might supply. But complete extraction without destruction of the sperm has not as yet been accomplished. From the present evidence it is reasonable to regard antifertilizin as involved in an initial step that facilitates fertilization but which may or may not be an essential part of the process. This initial step is evidently the reaction with fertilizin. In a previous article (Tyler, 1941), it has been shown that the presence of fertilizin on the egg serves as an aid to fertilization. Antifertilizin may, then, be considered to have a similar rôle in the case of the spermatozoön. For this purpose it is not effective when present in solution but only on the spermatozoön. Partial removal of the antifertilizin or its neutralization by means of an antiserum or by means of fertilizin results in a decrease or even complete suppression of the fertilizing power of the sperm. As an interpretation for the fertilization-facilitating action of fertilizin (Tyler, 1941) it was suggested that, in the form of a gel around the egg, it has a few superficial combining groups available which serve as the initial trap for the sperm but which do not neutralize all of the reacting groups (antifertilizin) on the sperm before the latter has reached the surface of the egg. On this basis the decrease in fertilizing power of the treated sperm may be interpreted to mean that, with fewer reacting groups available, there is more likelihood that they will all be neutralized before the spermatozoa reach the egg surface.

## Summary

1. Acidification of sea-urchin sperm suspensions to below pH 6 or brief heating above 30° C. liberates into the solution the substance termed antifertilizin which is defined by four manifestations of its reaction with fertilizin; (a) neutralization and (b) precipitation of the latter, (c) agglutination of eggs, (d) formation of precipitation membrane on egg jelly.

2. The treatment results in a marked decrease in the fertilizing power even when the time and intensity of exposure are not sufficient to immobilize the sperm.

3. The rate of oxygen consumption of sperm, that had been exposed to mild acid- or heat-treatment, was found to be very little affected in comparison with the effect on the fertilizing power. Short extrapolation permits the conclusion to be drawn that a considerable reduction in fertilizing capacity can be obtained with no reduction of activity of the spermatozoa.

4. Injection of antifertilizin solutions into rabbits results in the production of an agglutinin for the intact sperm. This shows that the substance is a complete antigen and supports the views that it is a protein and a component of the surface of the spermatozoön.

5. Fertilization is inhibited by antisera to antifertilizin.

6. Antifertilizin is considered to be concerned in an initial (perhaps essential) step in the union of the gametes whereby the spermatozoön is entrapped by the complementary, specific reacting substance, fertilizin, on the egg; and the above inhibition experiments are interpreted on the basis of a decrease in the number of reacting groups available on the spermatozoön.

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